

Expression of the vitellogenin gene in female and male sea urchin

(yolk protein/posttranslational modification/gene cloning/developmental regulation)

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ABSTRACT Expression of vitellogenin, the yolk protein precursor, is strictly regulated during development. In previous studies on a variety of organisms, vitellogenin gene expression has been shown to be restricted to one or two tissues in adult female animals. In this report we show that, in contrast, sea urchin vitellogenin is synthesized in both females and males. To identify sea urchin vitellogenin, we raised antibodies specific for the major yolk protein. We show here that a 155-kDa polypeptide, immunoprecipitable by the antibody to the major yolk protein, is synthesized in the intestines of female and male sea urchins and also in ovaries and testes. This 155-kDa polypeptide is converted to a 195-kDa vitellogenin in each of these tissues; further modification to yield the 180-kDa major yolk protein occurs only in the ovary. We have also identified a vitellogenin cDNA clone and used it to study vitellogenin mRNA production. An abundant 5.1-kilobase mRNA was found in the tissues containing vitellogenin. Our results suggest that vitellogenin may serve the following two functions in sea urchins: its classical role as a yolk protein precursor and an unidentified function required by adults of both sexes.

One of the most conspicuous aspects of oogenesis is the formation and storage of the yolk proteins, a process known as vitellogenesis (for review, see ref. 1). In most instances the yolk proteins present in eggs are cleaved from a common precursor called vitellogenin. In oviparous vertebrates, vitellogenin is produced in the female liver, transported in the blood, and selectively taken up into the growing oocytes. In invertebrates, vitellogenin is produced in the hermaphrodite intestine (nematodes) (2) and in female fat body (insects). In *Drosophila* the follicle cells of the ovary also produce vitellogenins (3). After synthesis, vitellogenin is secreted into the hemolymph or the pseudocoelomic fluid and eventually deposited in the oocytes. In all systems studied, the vitellogenins are cleaved or otherwise modified in the course of secretion, transport, or deposition in the growing oocytes.

The yolk proteins of sea urchin have been identified in the yolk platelets of several species (4–6). The most abundant yolk protein found in the eggs of four different species is a 180-kDa glycoprotein, designated major yolk protein (MYP) (4). Although the biochemical properties and function of sea urchin MYP have been studied, relatively little is known about its source. Harrington and Easton (4) presented evidence that an abundant 195-kDa polypeptide present in the coelomic fluid of adult female and also male sea urchins may be a precursor to MYP (4). This proposal was based on partial proteolysis mapping of MYP and the coelomic fluid protein in a one-dimensional NaDodSO₄ gel. The source of the putative precursor and its relationship to MYP were not further characterized.

Since vitellogenin is quite clearly absent from males in all previously described instances (nematodes, insects, and vertebrates) and since the relationship between the MYP and

the abundant coelomic fluid protein present in female and male sea urchins was not firmly established (4), we undertook the studies reported here. We have used an antibody specific for MYP from *Strongylocentrotus purpuratus* to demonstrate that the precursor present in the coelomic fluid of adults of both sexes is in fact vitellogenin. We have also determined that the gene (or genes) encoding vitellogenin is expressed in the intestines and gonads of adults of both sexes. This unique pattern of expression raises the intriguing possibility that, in the sea urchin, vitellogenin performs a function in addition to its role in vitellogenesis.

MATERIALS AND METHODS

Preparation of Anti-MYP Antiserum. A gel slice containing MYP was excised from a 5% polyacrylamide gel, rinsed in TE (10 mM Tris·HCl, 1 mM EDTA, pH 7.4), and homogenized in 2 ml TE. The homogenate was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into two rabbits. After 4 weeks, an equivalent booster injection with incomplete adjuvant was administered. Sera were prepared 3 weeks after the booster injection.

Isolation of Coelomic Fluid and Individual Tissues. The lantern structure (mouth) of an individual sea urchin was removed. Coelomic fluid (3–5 ml) was collected using a Pasteur pipet. Coelomocytes were removed by centrifugation of the coelomic fluid. Coelomic fluid (1 ml) was saved and stored at –80°C. The individual tissues (intestine, ovary, or testis) were removed from the opened sea urchin and small pieces (about 3 µl) were dissected for incubation *in vitro*.

Protein Synthesis in Isolated Tissues. Coelomic fluid (125 µl) was combined with an equal volume of culture medium [³⁵S]methionine (1 mCi/ml; 1 Ci = 37 GBq), 100 µM amino acid mixture lacking methionine, 50 mM sucrose, 2 mM glucose, and bovine serum albumin at 1 mg/ml in synthetic sea water} and incubated at 13°C for 6–8 hr. After incubation, coelomocytes were sedimented by centrifugation in an Eppendorf Microfuge, and the supernatant was removed. Immediately 60 µl of lysis solution [5% (wt/vol) NaDodSO₄, 10% (vol/vol) glycerol, 0.15 M Tris·HCl (pH 7.4), and 2 mM phenylmethylsulfonyl fluoride] was added to the pellet. The tube was boiled for 3 min, and unlysed cell debris was sedimented. Supernatant was saved and stored at –80°C. Dissected tissues were incubated in Eppendorf tubes containing 40–60 µl of culture medium at 13°C for 6–8 hr. Pieces were transferred to fresh Eppendorf tubes containing 60 µl of lysis solution after incubation and boiled for 3 min, and unlysed tissue was sedimented. Supernatants were saved and stored at –80°C for later analysis.

RNA Isolation. RNA was isolated according to the method of MacLeod *et al.* (7) except that the dissected tissues were ground up in liquid nitrogen before homogenization in 6.5 M guanidine hydrochloride. Poly(A)⁺ RNA was prepared by oligo(dT)-cellulose chromatography (8).

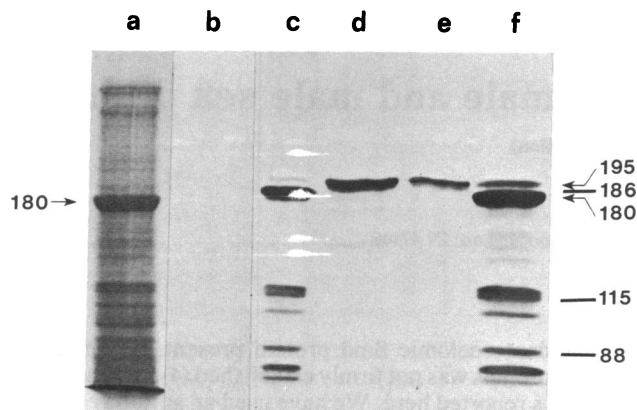


FIG. 1. Immunoblot analysis of protein preparations from *S. purpuratus*. Protein preparations from tissues from individual gravid sea urchins were separated on a 5% polyacrylamide/NaDodSO₄ gel according to Laemmli (12), as modified by Sharrock (13). Proteins were electrophoretically transferred to a nitrocellulose membrane (14). The blots were then labeled with anti-MYP antiserum via the avidin-biotin-peroxidase complex method (15). Lane a: Coomassie blue stain of total egg proteins. Lanes b-f: immunoblot analysis. Reaction of preimmune serum control with total egg proteins (lane b); reaction of immune serum with egg proteins (lane c); male coelomic fluid (lane d); female coelomic fluid (lane e); ovary (lane f). Molecular size standards in kDa are the *C. elegans* yolk proteins. The 186-kDa marker is equivalent to the yp170 described in the earlier papers (2, 13).

cDNA Library Construction. A total of 0.5 mg of intestinal poly(A)⁺ RNA was denatured with methyl mercury and fractionated on a 5–20% sucrose gradient as described by MacLeod *et al.* (7). High molecular weight RNA was recovered from the bottom third of the gradient by precipitation with ethanol. Double-stranded cDNA primed with oligo(dT) was made to the size-selected mRNA according to the methods of Wickens *et al.* (9) and Huynh *et al.* (10). The double-stranded cDNA was then cloned into λ gt10 using *Eco*RI linkers. The recombinant DNAs were packaged *in vitro* (11).

RESULTS

Antibody Specific to MYP. The proteins of unfertilized *S. purpuratus* eggs were separated on a 5% NaDodSO₄/polyacrylamide gel and the 180-kDa band, designated MYP (Fig. 1, lane a), was used for antibody preparation. Immunoblot analysis (Fig. 1) showed that the antiserum (lane c), but not preimmune serum (lane b), reacted primarily with the 180-kDa MYP as well as six lower molecular weight bands. These bands are normal constituents of the yolk glycoprotein complex and presumably are cleavage products of the MYP (5). In addition, periodic acid-Schiff (PAS) staining indicated that the six lower molecular weight bands are glycoproteins (data not shown). As a further test of the specificity of the antiserum, we carried out electron microscopic immunolocalization on unfertilized eggs. Fig. 2 shows that the

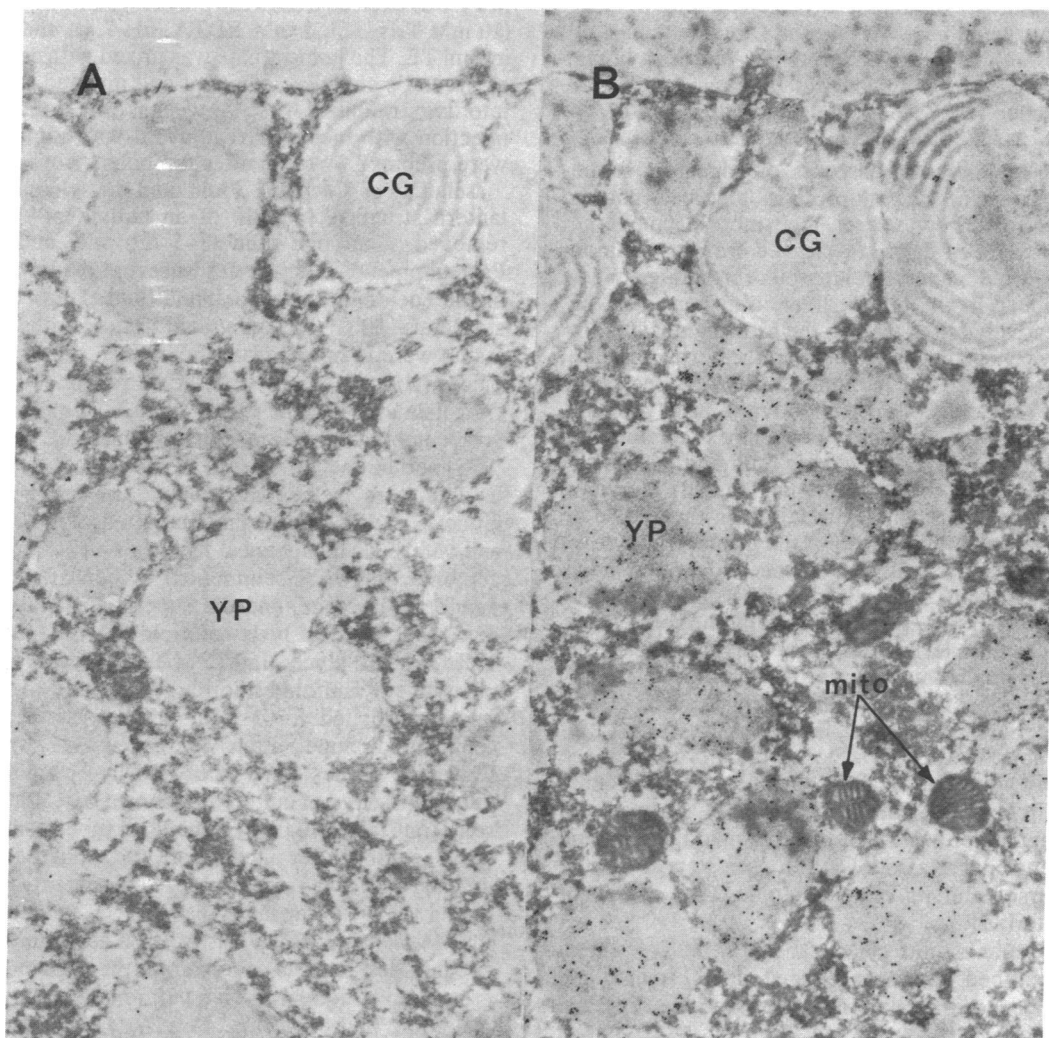


FIG. 2. Subcellular localization of MYP. Eggs were fixed with artificial sea water containing 0.5% glutaraldehyde and embedded in Lowacryl K4M. Sections were labeled with preimmune serum (A), and anti-MYP antiserum (B) by the immunogold method (16). CG, cortical granules; mito, mitochondria; YP, yolk platelets. ($\times 40,000$.)

anti-MYP antibody (Fig. 2B), but not preimmune serum (Fig. 2A), binds specifically to yolk platelets.

Vitellogenin Synthesis in Both Sexes of Adult Sea Urchin. The 195-kDa polypeptide found in coelomic fluid of adult sea urchins by Harrington and Easton (4) was hypothesized to be MYP precursor, based on peptide maps generated by partial proteolytic cleavage of the putative precursor and the egg MYP. To test this hypothesis, we subjected proteins from the coelomic fluid to immunoblot analysis. This analysis showed that the anti-MYP antiserum reacted with a single 195-kDa band from both male and female sea urchins (Fig. 1, lanes d and e), suggesting that this protein is the precursor to the components of the yolk platelets. We were also able to identify small amounts of the 195-kDa vitellogenin in ovary, in addition to the bands corresponding to MYP and the smaller yolk proteins (lane f).

To determine which adult tissues synthesize the vitellogenin, coelomocytes, testes, ovaries, and intestines were removed, labeled for 6–8 hr with [35 S]methionine *in vitro*, and labeled proteins were immunoprecipitated with anti-MYP antiserum and analyzed by NaDodSO₄/gel electrophoresis. Each tissue exhibited a characteristic and reproducible pattern of bands (Fig. 3). Intestines and gonads of both sexes showed a prominent immunoprecipitable band corresponding to the 195-kDa vitellogenin, whereas no protein could be immunoprecipitated from coelomocytes. We also observed a faint immunoprecipitable band of 155 kDa in intestines and

testis (Fig. 3). We present evidence below that this band most likely represents the initial translation product of the vitellogenin gene. We conclude that the MYP precursor, vitellogenin, is present in coelomic fluid, but is synthesized in intestines and gonads of both sexes of adult sea urchin. It is interesting to note that in the relatively long labeling period of this experiment (6–8 hr), none of the 195-kDa vitellogenin was converted to the 180-kDa MYP in the ovary. One possible explanation for this result is that only vitellogenin imported from the coelomic fluid is thus processed.

Identification of Vitellogenin mRNA in Intestine and Gonad.

To identify the vitellogenin message, RNA was extracted from embryos and from four adult tissues (coelomocytes, testis, ovary, and intestine). When these RNA preparations were translated in a rabbit reticulocyte lysate, and the products were resolved on a 7.5% NaDodSO₄/polyacrylamide gel, no discrete band of similar abundance was observed to migrate with the 195-kDa vitellogenin band (Fig. 4). Immunoprecipitation of the *in vitro* translation products with anti-MYP antiserum, however, revealed a prominent band of 155 kDa present in translation products of intestine and gonad but not in those of coelomocytes or embryos (Fig. 4). Based on the reactivity of this band with the antiserum,

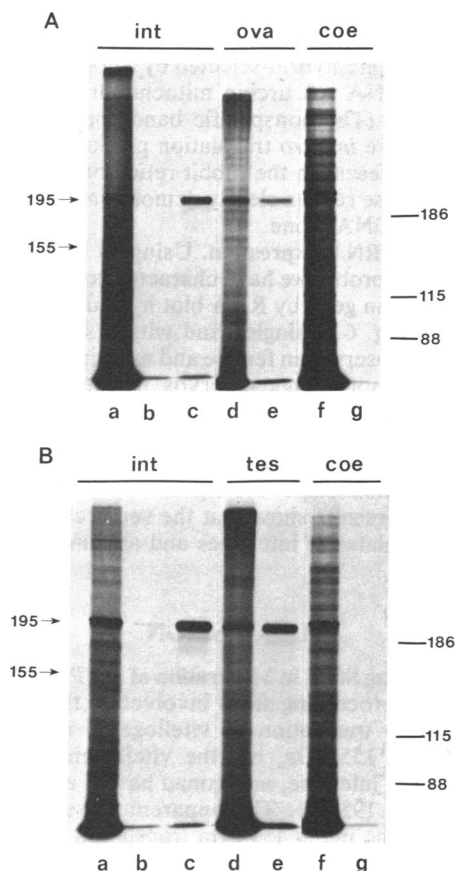


FIG. 3. Immunoprecipitation of vitellogenin made in isolated tissues. Dissected tissues from female (A) or male (B) were labeled. Immunoprecipitation of equal counts (cpm) of labeled products was performed using protein A-Sepharose (17). Proteins of the labeled homogenate (lanes a, d, and f) or antigen-antibody complex (lanes b, c, e, and g) were resolved on a 5% polyacrylamide/NaDodSO₄ gel. Lanes: a and c, intestine (int); b, precipitation of intestine with preimmune serum; d and e, gonad (ova, ovary; tes, testis); f and g, coelomocytes (coe). Size markers are in kDa.

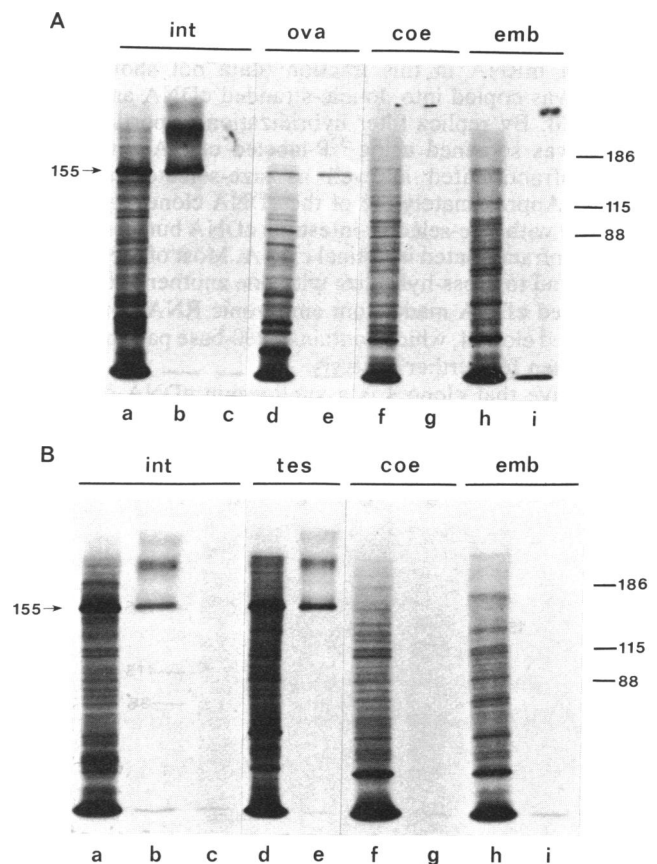


FIG. 4. *In vitro* translation of mRNA isolated from various tissues. RNAs from female (A) or male (B) were translated in a rabbit reticulocyte lysate purchased from Bethesda Research Laboratories, according to the procedure provided by the supplier. Each 30- μ l reaction mixture contained 7 μ g of total RNA. Immunoprecipitation of *in vitro* translation products was then carried out (17). *In vitro* translation products (lanes a, d, f, and h) and immunoprecipitation products (lanes b, c, e, g, and i) were separated on a 7.5% polyacrylamide/NaDodSO₄ gel. Lanes: a and b, intestine (int); c, preimmune control of intestine; d and e, gonad (ova, ovary; tes, testis); f and g, coelomocytes (coe); h and i, embryos (emb). The smears present at the top in A, lane b, and B, lanes b and e, are due to nonspecific trapping by Sepharose beads contaminating the samples. Size markers are in kDa.

this result suggests that the 155-kDa polypeptide is a precursor to vitellogenin. Consistent with this interpretation, we found both 155- and 195-kDa immunoreactive proteins were synthesized by intestines and gonads (Fig. 3). The relationship between the 155-kDa polypeptide and the 195-kDa vitellogenin was further investigated in a competition experiment. We found that binding of the 155-kDa *in vitro* translation product to the antibody could be completely inhibited by addition of the 195-kDa vitellogenin (data not shown). Hence, it appears that the same antibodies are recognizing both polypeptides. These observations suggest that the 155-kDa polypeptide is synthesized in the intestines and gonads of both sexes of adult sea urchins and is modified posttranslationally, probably by glycosylation (5, 6), in the tissues in which it is produced. Since we found mature MYP only in the ovary and eggs (Fig. 1, lanes c and f; and unpublished observation), it appears that the final maturation step leading to MYP formation occurs only after uptake into the oocytes.

Isolation of cDNA Clones Encoding Vitellogenin. To clone the gene(s) for vitellogenin, we constructed an intestinal cDNA library. Intestinal poly(A)⁺ RNA from a population of adult sea urchins was fractionated on a sucrose gradient, and the mRNA from the bottom one-third of the gradient was pooled. *In vitro* translation indicated that the vitellogenin mRNA was enriched by a factor of 4–6. It was the most abundant mRNA in this fraction (data not shown). The mRNA was copied into double-stranded cDNA and cloned into λ gt10. By replica filter hybridization, a portion of the library was screened using ³²P-labeled cDNA synthesized from unfractionated as well as size-selected intestinal mRNA. Approximately 6% of the cDNA clones hybridized intensely with size-selected intestinal cDNA but less intensely with unfractionated intestinal cDNA. Most of these clones were found to cross-hybridize with one another but not with ³²P-labeled cDNA made from embryonic RNA. One clone, designated clone 4, which contains a 980-base pair (bp) insert, was chosen for further analysis.

To prove that clone 4 is a vitellogenin cDNA clone, we carried out a hybrid selection experiment. Clone 4 DNA was immobilized on nitrocellulose filters and tested for its ability

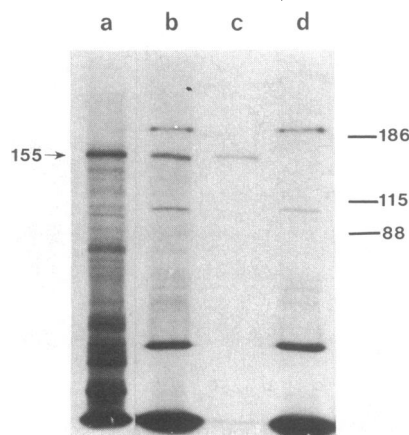


FIG. 5. *In vitro* translation of hybrid-selected mRNA. Hybrid selection of vitellogenin mRNA was carried out by the method of Ricciardi *et al.* (18) except that poly(A) was introduced in the hybridization mixture to a final concentration of 0.1 mg/ml. Adult intestinal poly(A)⁺ RNA (15 μ g) was hybridized to 100 μ g of immobilized clone 4 DNA. The ³⁵S-labeled products translated from unselected intestinal poly(A)⁺ RNA (lane a) and from poly(A)⁺ RNA selected by hybridization to clone 4 DNA (lane b) or to a λ gt10 clone containing cDNA encoding a sea urchin mitochondrial rRNA (lane d) were analyzed by 7.5% NaDodSO₄ gel electrophoresis. The hybrid-selected 155-kDa polypeptide was immunoprecipitated by anti-MYP antiserum (lane c). Size markers are in kDa.

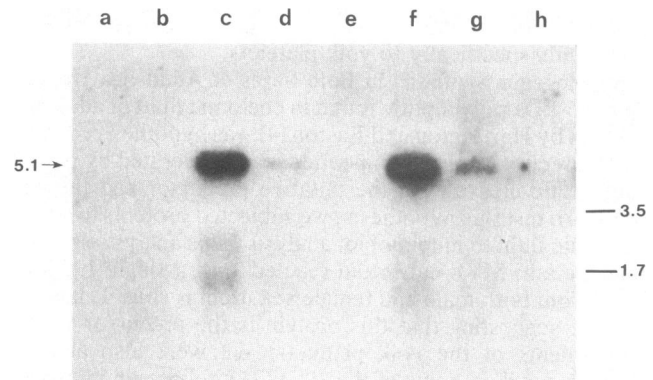


FIG. 6. Stage- and tissue-specific expression of vitellogenin mRNA. Total RNA (5 μ g), isolated from different sources, was subjected to electrophoresis through a 1% methyl mercury gel (19), blotted onto a nitrocellulose filter, and probed with a ³²P-labeled vitellogenin cDNA insert subcloned from clone 4 into M13. Lane a, eggs; lane b, embryos; lane c, female intestine; lane d, ovary; lane e, female coelomocytes; lane f, male intestine; lane g, testis; lane h, male coelomocytes. Size markers are in kilobases.

to hybrid-select vitellogenin mRNA. As shown in Fig. 5, lane b, *in vitro* translation of the hybrid-selected mRNA resulted in the synthesis of a single polypeptide of 155 kDa. This corresponds in size to the unmodified vitellogenin precursor (Fig. 5, lane a). Furthermore, Fig. 5, lane c shows that the hybrid-selected 155-kDa polypeptide was specifically immunoprecipitated by anti-MYP antiserum. No *in vitro* translation products were hybrid-selected by a control λ gt10 clone containing a cDNA sea urchin mitochondrial rRNA insert (Fig. 5, lane d). (The nonspecific bands present in Fig. 5, lanes b and d, are *in vitro* translation products from endogenous mRNA present in the rabbit reticulocyte lysate; data not shown.) These results clearly demonstrate that clone 4 is a vitellogenin cDNA clone.

Vitellogenin mRNA Expression. Using an M13 subclone of clone 4 DNA as probe, we have characterized the expression of the vitellogenin gene by RNA blot hybridization analysis. As shown in Fig. 6, a single band with a size of about 5.1 kilobases was observed in female and male intestines, testes, and ovaries but not in eggs, embryos, or coelomocytes. This result is consistent with the *in vitro* translation and immunoprecipitation experiments. We also probed RNAs from three different stages of embryogenesis: early blastula, late gastrula, and prism stage. No signal was detected (data not shown). These results show that the sea urchin vitellogenin mRNA accumulates in intestines and also in gonads, but at lower levels.

DISCUSSION

Two Processing Steps in Maturation of MYP. It appears that there are two processing steps involved in the formation of MYP. *In vitro* translation of vitellogenin mRNA gives a polypeptide of 155 kDa, but the vitellogenin identified in coelomic fluid, intestine, and gonad has an apparent molecular weight of 195,000. The apparent increase of 40 kDa suggests that the initial 155-kDa translation product is subjected to significant modification. To support this interpretation, our analysis of the *in vivo* synthesis products from labeled tissues shows that besides the 195-kDa vitellogenin band a minor band of 155 kDa can also be immunoprecipitated by the antiserum; no immunoreactive proteins smaller than the 155-kDa band were observed. Furthermore, Armant *et al.* (6) observed a shift of 35 kDa after the endoglycosidase H digestion of MYP from another sea urchin species, *Arbacia punctulata*, indicating that the MYP is highly glycosylated. Similar posttranslational modifications have

been reported in other systems. Studies on the insulin receptor suggest that α and β subunits are synthesized as single polypeptides of 180 and 190 kDa, respectively, which undergo glycosylation, resulting in the apparent addition of 30 kDa to each (20–23). Also, human epidermal growth factor receptor has been shown to be a 175-kDa glycoprotein, 37 kDa of which is due to oligosaccharide side chains (24). Together, these observations suggest that the 155-kDa initial translation product reported here is the precursor to the vitellogenin and is modified posttranslationally to give rise to mature vitellogenin.

A second processing step results in an apparent shift of the 195-kDa vitellogenin to the 180-kDa mature MYP found in eggs. This final step only occurs in ovary (Fig. 3, lanes b and f), presumably in the oocytes.

Regulation of Vitellogenin Synthesis. In all respects but one, vitellogenin production in the sea urchin is like that described in other systems. First, it is regulated at the level of RNA accumulation. Second, vitellogenin mRNA is found only in adult intestine, ovary, and testis. However, in other systems analyzed (vertebrates, insects, and nematodes), expression is limited to females. In sea urchins large amounts of vitellogenin are produced in both females and males. In females vitellogenin accumulates in the coelomic fluid and also is transported to oocytes, where it is processed to MYP. In males, vitellogenin accumulates in the coelomic fluid. Based on NaDodSO₄ gels of total protein, we estimate that greater than 50% of the protein in coelomic fluid of both sexes is vitellogenin (unpublished observations). The presence of such large quantities of this protein raises the intriguing possibility that it performs some function in addition to its role as a precursor to yolk protein, perhaps serving either as an analog to the serum albumin of vertebrates, as a carrier protein, or as a store for amino acids. Alternatively, vitellogenin may perform no function in the male; rather, its presence may result from an as yet undescribed sex determination mechanism in sea urchin, one that may not lead to sexual dimorphism in gene expression.

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